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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/62 // C12N 15/16
A61K 39/385

A1

(11) International Publication Number: WO 90/02187

(43) International Publication Date: 8 March 1990 (08.03.90)

(21) International Application Number: PCT/EP89/01013

(22) International Filing Date: 29 August 1989 (29.08.89)

(30) Priority data:
246,558 30 August 1988 (30.08.88) US

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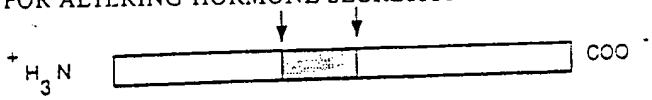
(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent)

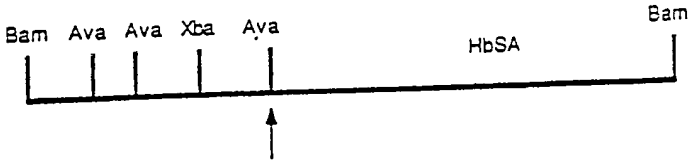
Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: RECOMBINANT FUSION PROTEINS FOR ALTERING HORMONE SECRETION

(57) Abstract

A recombinant fusion protein, resulting from the expression of two genes regulated by a single promoter, having the effect of altering hormone secretion when administered in a therapeutically effective dose. One gene codes for the production of a highly antigenic, hydrophilic protein, such as hepatitis B surface antigen, and the other gene codes for a peptide which alone is not substantially antigenic, such as luteinizing hormone releasing hormone.





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RECOMBINANT FUSION PROTEINS FOR ALTERING HORMONE SECRETION

Field of Invention

This invention relates to the production of fusion proteins produced by recombinant DNA technology and used as active immunogens against peptides which are not inherently antigenic. By combining the gene for a highly antigenic protein, such as the hepatitis surface antigen (HBsAg), and the cDNA encoding for a protein, portion of a protein or peptide which is not inherently antigenic, a fusion protein may be expressed. Following injection of this highly antigenic fusion protein, polyclonal antibodies are produced that are directed against both the highly antigenic protein and the protein of interest. Thus, this technology provides a method to immunoneutralize endogenously produced hormones and could be used to induce an infertile state or inhibit the hormone dependent growth of rapidly multiplying tissue.

Background of the Invention

As a result of the pioneering work of Guillemin, Schally and coworkers during the late 1960's, it has become evident that the hypothalamic decapeptide, luteinizing hormone releasing hormone (LHRH) is intimately involved in the initiation and maintenance of reproductive competence in all mammals. Disruption or destruction of neuronal pathways involved in the synthesis of LHRH or its transport to the capillary network that provides humoral communication between the hypothalamus and pituitary results in a

reduction in gonadotropin synthesis and release and infertility (Kalra and Kalra, Endocrine Reviews 4:311, 1983).

Similarly, reductions in LHRH-pituitary interactions may be elicited through the administration of long-acting competitive antagonists of LHRH. These synthetic peptides bind to the pituitary LHRH receptor but do not stimulate post-receptor intracellular activities. Due to their unusually long receptor occupancy, caused by their resistance to proteolytic inactivation, these peptides induce a state of pituitary insensitivity to LHRH due to down regulation of LHRH receptor number. This pharmacologic reduction in pituitary stimulation by LHRH antagonists results in a reduction in gonadotropin release and infertility (Labrie et al [eds] LHRH and its analogs. Amsterdam: Elsevier Scientific Publishers. 1984).

Another method employed to disrupt LHRH-pituitary interactions has been the production of antibodies against LHRH. Active LHRH immunization has been accomplished in a number of species including the rat (Fraser et al; J. Endocrinology 63:399, 1974), rabbit (Arimura et al; Endocrinology 93:1092, 1973), ewe (Clarke et al: J. Endocrinol 78:39, 1978) and rhesus monkey (Chappel et al; Biol. Reprod. 22:333, 1980). In all species, active immunization against LHRH results in a loss of reproductive function characterized by low serum concentrations of gonadotropins and gonadal steroids and impairments in gametogenesis. Due to the extremely short half-life of LHRH in an animal (less than five minutes), most active immunization has been performed with LHRH conjugated to much larger molecules, such as bovine serum

albumin and presented in Freund's Complete Adjuvant, to induce an immune response. Immunoneutralization of hypothalamic LHRH can be employed as a routine procedure to induce an infertile state characterized by low circulating levels of gonadotropins and gonadal steroids and anovulation or azospermia.

The production of antibodies against LHRH by active immunization is uncomfortable, if not painful, to the animal. Although this method would induce a state of infertility and reduce behavioral heat (due to low circulating gonadal steroids) it could not be employed as a pet contraceptive.

There is a serious over-population of cats and dogs in the United States which is of great concern to the general public, animal welfare organizations, veterinarians, and health officials. In the feline population, the reproductive rate has been reported to be almost twice the rate at which homes become available for these animals (Shreider and Vaida. J Am Vet Med Assoc. 166 481, 1975). To halt the growth of the feline population, approximately 88% of reproductively competent females should be spayed (Nassar and Masier, Am J Vet Res. 43:167, 1982). Approximately 20% of the dog population in the United States passes through an animal shelter each year and of that, approximately 80% of these animals are destroyed. The cost of impounding and destroying unwanted cats and dogs ranges from $125 to $500 million per year (Am. Humane Society: Proceedings on Ecology of the Surplus Cat and Dog Problem).

At present, the only solutions to the problem of unwanted pregnancies in the dog and cat population are complete confinement and surgical gonadectomy. Neither method may be considered effective as a means of birth control. Confinement does not reduce behavioral problems associated with heat and neutering clinics do not reach sufficient numbers of animals to alleviate the over-population problem. Costs of skilled professionals to perform the surgery, anesthesia, antibiotics and postoperative care are beyond the means of many pet owners.

It is an object of the present invention to provide a recombinant fusion protein or vaccine which provides an effective, safe, available and reversible means of fertility control. A vaccine that is easily administered is likely to supplant surgical sterilization while providing an effective contraceptive for dogs, cats and other mammals.

Pregnant cows typically do not exhibit an attractive feed to meat conversion since the developing fetus requires great nutritional requirements. As a result, feed lot owners generally pay substantially less for a cow in which the pregnancy status is unknown or unconfirmed. A certain percentage of cows enter the feed lot pregnant and the fetus represents waste. Thus, a method to induce an infertile state in cows 6-10 months prior to shipment is considered to be a viable, valuable product. If the farmer could guarantee that his cattle are "barren" he could command a higher price for his animals.

It is, therefore, another object to provide a recombinant, injectable product which would result in inhibition of ovulatory cyclicity in feedlot heifers.

There are many examples of human hormonally-mediated tissue hyperplasia. In the male, it is well known that prostatic carcinoma (BPH) is dependent upon testicular testosterone for continued growth. It would be advantageous to reduce serum levels of gonadotropins to inhibit gonadal steroid secretion and thus induce involution of hyperplastic tissue. It has recently been reported that a long-acting LHRH antagonist may be used in the treatment of prostatic hyperplasia (Peters and Walsh, New England Journal Medicine 317:599, 1987). The mechanism of action of this treatment is the inhibition of LH and FSH secretion by down-regulation of pituitary LHRH receptors. This results in an inhibition of gonadotropin secretion which causes a fall in testosterone production. A reduction in serum testosterone causes a regression of the prostatic hyperplasia. The authors note, however, that inhibition of LH secretion as elicited by injection of an LHRH antagonist would be required for prolonged periods of time, thus necessitating repeated administration.

It is yet another object of the present invention to avoid injections of LHRH antagonists to inhibit gonadotropin secretion, immunoneutralization of endogenous LHRH.

It is still a further object of the present invention to provide a method to make any substantially non-antigenic peptide highly immunogenic, preferably without the necessity of use of adjuvants such as Freund's Complete Adjuvant.

Summary of the Invention

In accordance with the principles and objectives of the present invention, there are provided methods for creating fusion proteins that may be employed as potent immunogens. Ideally, this method of the present invention employs molecular biology techniques to fuse cDNAs together, and to place them into a eukaryotic expression vector for the production of unique fusion proteins. The majority of this fusion protein is a highly antigenic and potent immunogen. It should have substantial hydrophilicity. Good examples of such immunogens are coat proteins such as the hepatitis B surface antigen. An oligonucleotide coding for the peptide of interest, against which it is desired to raise antibodies, such as the luteinizing hormone releasing hormone molecule, is placed in one or more regions of the protein, preferably in the one or more regions having the highest hydrophilicity. Injection of the resultant protein, most preferably an LHRH-HBsAg fusion protein, in therapeutically effective concentration, will result in the development of antibodies against the entire molecule. A portion of these antibodies will be directed against the peptide of interest. Most preferably, when the peptide is LHRH, sufficient titres of antibodies against LHRH will be provided thereby preventing hypothalamic-pituitary communication and inducing an infertile state. This immunogen may also be advantageously employed in a variety of commercial or therapeutic settings including an animal anti-fertility vaccine and treatment for benign prostatic hypertrophy.

The fusion proteins of the present invention are sufficiently immunogenic so as to preclude the necessity of use of adjuvants or multiple injection, neither of which are well tolerated by the subject.

Brief Description of the Drawings

Figure 1. Illustrates the insertion of gonadotropin releasing hormone sequences within the exposed hydrophilic region of HBsAg protein. At the top is a diagram of the HBsAg protein in which the site containing the LHRH decapeptide is stippled. Below that is the AvaII site along the cDNA that codes for HBsAg in which the cDNA for LHRH is ideally inserted. The figure at the bottom represents a viral coat particle with LHRH inserted between the coat protein sequences.

Figure 2. Illustrates a synthetic DNA linker that will be inserted into the AvaII site of the HBsAg (see Fig. 1). This linker provides a flexible insertion site for oligonucleotide sequences after amino acid 119 (glycine) of the HBsAg. It results in the insertion of three amino acids before the resumption of the HBsAg sequence at the proline residue (position 120). The LHRH fragment is preferably inserted between the SmaI and BglII site of the linker. Addition of the linker will maintain the proper reading frame (see Fig. 3).

Figure 3. Shows the nucleotide sequence for the LHRH peptide to be inserted in the synthetic linker within the HBsAg cDNA. To maintain the proper reading frame, two additional nucleotides (GG) have been added to the 3' end of the oligonucleotide which will add a glycine (GGG) immediately following the inserted LHRH peptide and maintain the proper reading frame. Note the GG added to the 3' end to maintain the proper reading frame when inserted into the synthetic DNA linker (Figure 2). We have also added the BglII overhang for proper insertion into the cut linker.

Figure 4. Diagrams the scheme used to insert HBsAg-LHRH fusion into mouse metallothionein promoter (CL28 vector) and subsequent insertion of the BPV genome. The plasmid BPV-HL is advantageously used to transform C127 cells.

Detailed Description of the Invention and Best Mode

The gene encoding for the hepatitis B surface antigen has already been cloned and expressed (Hsiung et al., J. Mol. Appl. Gen. 2:497, 1984). This gene is advantageously expressed in our mammalian expression system. The DNA encoding the coat protein was cloned into the mouse metallothionein gene, just a few base pairs from the site of transcription initiation. All of the signals that regulate the expression of the inserted gene (for coat protein) in mammalian cells are supplied by the mouse gene, whose coding sequences and poly A addition signals lie downstream from the insert. The surface antigen protein is secreted into the

medium where it is found in the form of particles. The recombinant product (i.e., surface antigen without nuclear material) has been shown to be highly immunogenic in guinea pigs.

From the DNA sequence, the amino acid sequence of the hepatitis antigen was determined. This sequence was analyzed for the region of the molecule that would be expected to have the most hydrophilic properties. Into that region (identified by the AvaII site in Figure 1) was cloned a short oligonucleotide sequence that provides unique cloning sites (Figure 2). Into this region, cDNA sequences encoding for specific proteins may be inserted either by blunt end ligation at a unique SmaI site or at the BglII site (see Figure 2).

Using the published structure of the hypothalamic gene (Seeburg and Adelman: Nature 311:666, 1984: Figure 3) but modified as described above, the oligonucleotide encoding LHRH was synthesized on an Applied Biosystems 380-A automated DNA synthesizer using the solid phase synthetic methods developed by Caruthers et al (Genetic Engineering 4:1-17, 1982). The oligonucleotide was cleaved from the solid support using concentrated ammonium hydroxide. Purification was achieved using preparative gel electrophoresis. The purified oligonucleotide was polynucleotide kinase and analyzed by gel electrophoresis for size and purity.

The LHRH oligonucleotide was inserted into the SmaI and BglII cloning site by the following method: The LHRH oligonucleotide was synthesized (as shown in Figure 3) to contain a 3' blunt end and a 5' CTAG overhang. Following digestion of the HBsAg DNA (containing the synthetic insert) with Sma I and BglII, the LHRH

DNA was inserted. The resultant construction was named HBsAg-LHRH. Synthetic BamHI linkers were added to each terminus of the HBsAg-LHRH cDNA and inserted into the vector CL28/Bam in the proper orientation. CL28/Bam obtained from Dr. Dean Hamer (at the NIH) contains a 3.8 kbp mouse DNA fragment that includes the entire metallothionein gene, including upstream control elements, inserted into the EcoRI site of the bacterial vector pBR322. CL28/Bam is a derivative of pJYMMT(E) (Hamer, Dean et al, J. Mol. Appl. Genet. 1, 4, 273-288 (1982)) which is identical to pJYMMT(E) except for the removal of the SV40 sequences. A unique BglII site located just after the transcriptional initiation region of the mouse gene allows the insertion of foreign genes as BamHI fragments which have the same 5' overhang (GATC) as the cut BglII site. Following the insertion of the HBsAg-LHRH fusion gene into CL28/Bam and endonuclease verification of orientation by the proper restriction pattern (with respect to the murine promoter) the entire genome of Bovine Papilloma virus (BPV) was inserted as a 7.8 kbp BamHI-SalI fragment. The final construction was verified by examination of diagnostic restriction enzyme digestion patterns on both agarose and polyacrylamide gels. A schematic representation of this method is provided in Figure 4.

Thus, the resultant hepatitis B surface protein contained an LHRH sequence within a hydrophilic region of the molecule.

To produce a cell line that expresses the fusion HBsAg/LHRH protein, 10µg of the purified BPV-HBsAg-LHRH cDNA was added to 0.5 ml of a 250mm $CaCl_2$ solution containing 10µg salmon

sperm DNA as a carrier. The mixture was bubbled into 0.5ml of 28mM NaCl, 50mM HEPES and 1.5mM sodium phosphate. The calcium phosphate precipitate was allowed to form for 30-40 minutes at room temperature.

Twenty-four hours prior to transfection, 5 x 10^5 mouse C127 cells were placed into 100mM petri dish. Immediately before adding the exogenous DNA, cells were fed with fresh medium (Dulbecco's modified Eagle's medium plus 10% fetal calf serum). One ml of calcium phosphate precipitate were added to each dish (10ml) and the cells incubated for 6-8 h at 37°C.

Medium was aspirated and replaced with 5ml of 20% glycerol in phosphate buffered saline (pH = 7.0) for 2 min. at room temperature. Cells were washed with phosphate buffered saline, fed with 10ml medium and incubated at 37°C. After 20-24 hours, medium was changed and cells refed every 3-4 days.

After 14-21 days, foci of transformed cells, characterized by their altered morphology, were identified and transferred to T-25 flasks to begin line expansion. Medium obtained from the cell lines was tested for presence of HBsAg and LHRH immunoactivity. Tissue culture medium was clarified by low speed centrifugation and HBsAg particles were sedimented at 42,000 rpm (200,000 xg) for 4 hours. Pellets were resuspended in a small volume of phosphate-buffered saline and assayed along with starting material and supernatants for HBsAg activity with a commercial radioimmunoassay (RIA) purchased from Clinical Assays, Inc. The culture medium supernatants and resuspended pellets were analyzed for LHRH immunoactivity with a radioimmunoassay utilizing LHRH antiserum

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/EP 89/01013

SA 30575

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 14/09/89
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0175261	26/03/86	JP-A- 61129135	17/06/86
		US-A- 4722840	02/02/88
US-A- 3963691	15/06/76	NONE	
EP-A2- 0219106	22/04/87	AU-D- 63997/86	30/04/87
		JP-A- 62099398	08/05/87
EP-A1- 0131363	16/01/85	GB-A-B- 2140810	05/12/84
		WO-A- 84/04756	06/12/84
		JP-T- 60501391	29/08/85
		DE-A- 3477810	24/05/89

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purchased from Arnel Antibodies, Inc. The LHRH for iodination and the reference preparation were purchased from Sigma Fine Chemicals, Inc., and the insoluble protein A (to pellet antigen-antibody complexes) was obtained from Sigma Fine Chemicals, Inc.

Following these procedures, cell lines expressing the fusion protein were produced and readily identified by standard screening methods. With a few milligrams of purified material, the appropriate dose to elicit an infertile state in male rabbits or other animals may be determined with a titration type procedure. For example, male rabbits injected with 0.5, 1 or 5 mg of the protein coupled with blood sample collection will allow determination of serum gonadotropin and testicular steroid levels as well as anti-LHRH antibody titres. Similarly, appropriate boosting intervals may also be determined.

From the foregoing, those skilled in the art will readily determine that numerous modifications can be made to the fusion genes and gene products such as the selection of the antigenic gene or the protein gene whose activity is to be immunologically suppressed, without departing from either the spirit or scope of the present invention. For example, highly immunogenic proteins other than HBsAg may be used, such as other hydrophilic coat proteins or the hepatitis B core antigen. Furthermore, those skilled in the art will understand that peptides other than LHRH may be used. While a similar method has been reported for presenting antigenic epitopes to the immune system, in Australian patent 69792/87, antigenic epitopes are always used, such as viral, bacterial or protozoan epitopes. It has not before been

suggested to use non-antigenic peptides in such a fusion protein in order to be able to induce the production of antibodies against such peptides without the disadvantages of prior art haptenization techniques.

Examples of substantially non-antigenic peptides are peptides which do not substantially differ in structure across species or any other peptide or protein which, when injected into a foreign animal, does not cause any substantial titre of antibodies to be raised against it. Besides LHRH, there may be mentioned growth hormone releasing hormone, adrenocorticotropin hormone, parathyroid hormone, inhibin, and subunits of gonadotropins. Portions of or slightly modified versions of such peptides may also be used.

What is claimed is:

1. A recombinant fusion protein, resulting from the expression of two genes regulated by a single promoter, wherein the first of said genes codes for the production of a highly antigenic, hydrophilic protein and the second of said genes codes for a peptide which alone is not substantially antigenic, or for a portion of such a peptide.

2. The fusion protein of Claim 1 wherein the fusion protein is highly antigenic and causes the production of antibodies which include antibodies against said substantially non-antigenic peptide.

3. The fusion protein of Claim 2 wherein said peptide comprises a hormone and the antibodies produced can immunoneutralize the same hormone endogenously produced.

4. The fusion protein of Claim 1 wherein said highly antigenic, hydrophilic protein is hepatitis surface antigen.

5. The fusion protein of Claim 4 wherein the second gene encodes for a protein having hormonal activity substantially similar to the activity of an endogenously produced hormone.

6. The fusion protein of Claim 5 wherein said second gene encodes for luteinizing hormone releasing hormone.

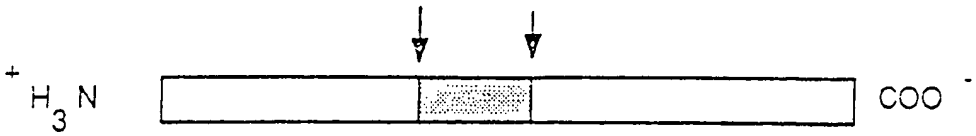
7. The fusion protein of claim 1 wherein said second gene is inserted into one or more regions of said first gene, each of said regions encoding a highly hydrophilic region of said protein.

8. DNA encoding for the fusion protein of Claim 1 comprising said two genes, a transcriptional initiation region, a promoter and an expression vector, said DNA capable of transforming mammalian cells to express the fusion protein .

9. The DNA of Claim 8 comprising DNA encoding for HBsAg-LHRH protein fused with a mouse metallothionein promoter and a bovine papilloma virus genome in proper sequence and reading frame whereby a luteinizing hormone releasing hormone protein is expressed having antigenic characteristics.

10. The DNA of claim 8 wherein said second gene is inserted into one or more regions of said first gene, each of said regions encoding a highly hydrophilic region of said protein.

FIG 1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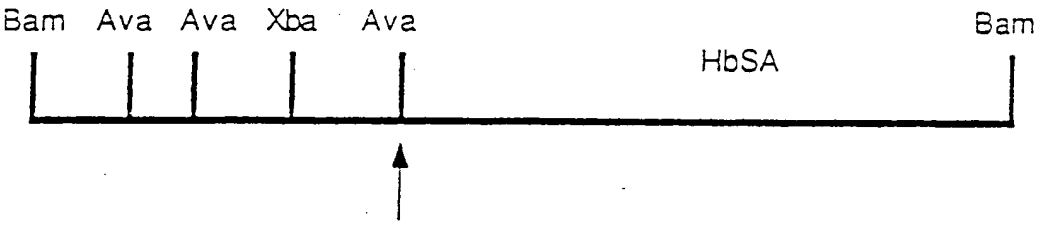
COO -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Ava
Ava
Xba
Ava
HbSA
Bam


Bam
Ava
Ava
Xba
HbSA + LHRH
Bam

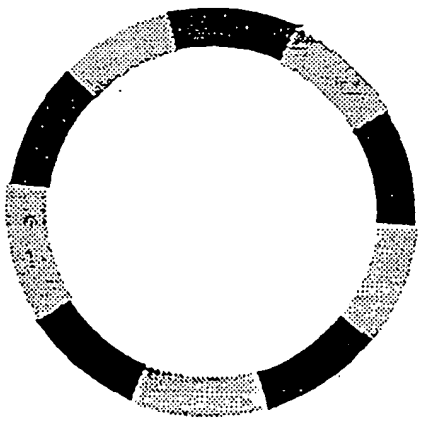
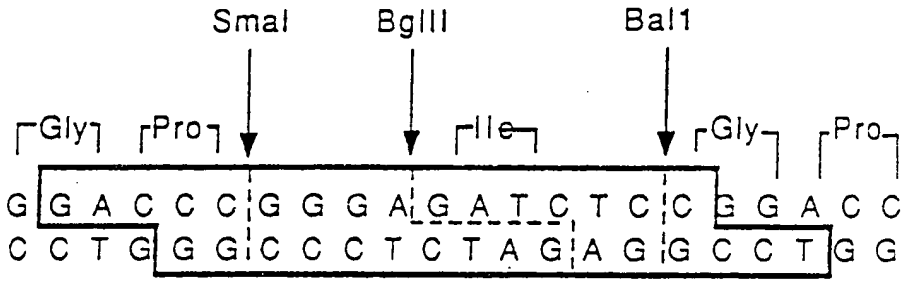


FIG 2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BglII
BalI
Gly
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Pro
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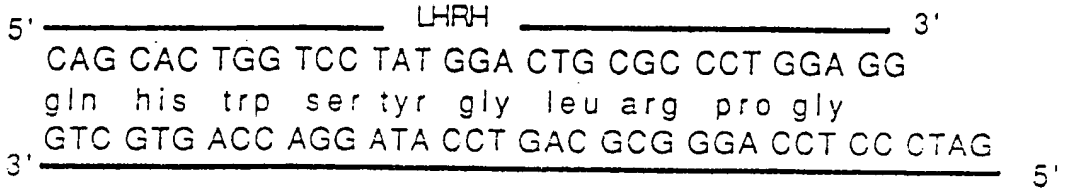
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LHRH
3'
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gln his trp ser tyr gly leu arg pro gly
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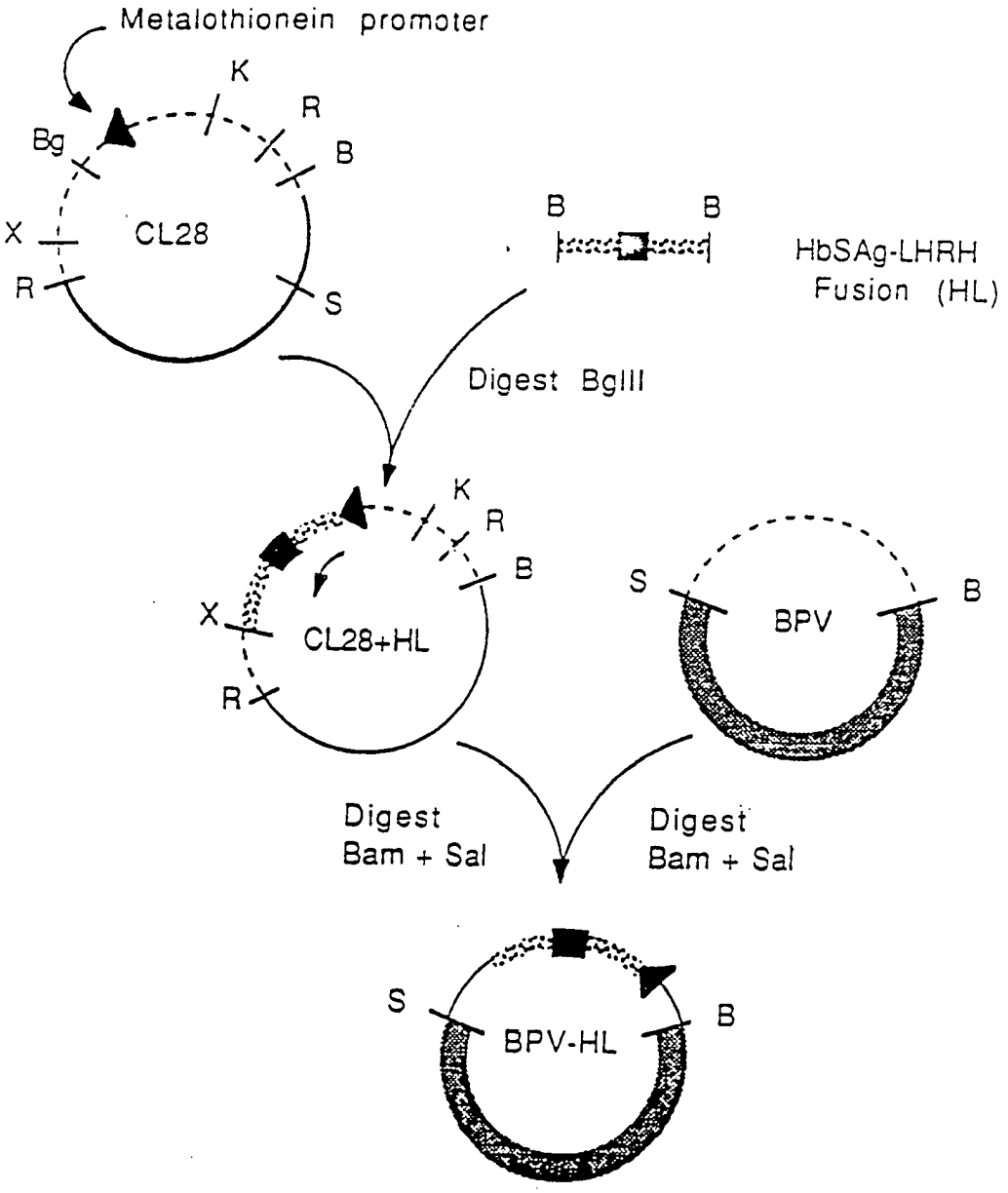
3'
5'

FIG 4


Metalothionein promoter
K
R
B
Bg
X
CL28
R
S
B
B
HbSAg-LHRH
Fusion (HL)
Digest BglII
K
R
B
X
CL28+HL
R
S
B
BPV
Digest
Bam + Sal
Digest
Bam + Sal
S
B
BPV-HL

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 89/01013

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 15/62, 15/16 // A 61 K 39/385

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC5	C 12 N; C 12 P; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A2, 0175261 (CHIRON CORPORATION) 26 March 1986, see the whole document	1,2,4,7, 8,10
Y		3,5,6,9
	--	
Y	US, A, 3963691 (HOFFMAN ET AL) 15 June 1976, see the whole document	3,5,6,9
	--	
X	EP, A2, 0219106 (F. HOFFMANN-LA ROCHE & CO) 22 April 1987, see the whole document	1,2
	--	

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
3rd November 1989	10. 01. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	EP, A1, 0131363 (CELLTECH LIMITED) 16 January 1985, see the whole document -- --------	1-3,5,7, 8